MOLECULAR AND CELLULAR BIOLOGY, Apr. 1987, p. 1530–1534
0270-7306/87/041530-05$02.00/0
Copyright © 1987, American Society for Microbiology
Vol. 7, No. 4

NOTES

Heat-Inducible Human Factor That Binds to a Human hsp70 Promoter

ROBERT E. KINGSTON,* THOMAS J. SCHUETZ, AND ZOIA LARIN

Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, and Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received 3 September 1986/Accepted 18 December 1986

A factor found in nuclear extracts of human cells bound to the heat shock element of a human heat shock protein 70 gene. The level of this factor was significantly increased after heat shock. This induction was rapid and was not blocked by cycloheximide, suggesting that an initial event in the response of a human cell to heat is the activation of a preexisting regulatory factor.

Organisms from bacteria to humans respond to high temperatures by synthesizing a set of protective proteins (20). In many organisms, the heat shock response occurs via an increase in the rate of transcription of a set of heat shock genes. A promoter sequence responsible for this induction has been identified and is conserved from *Drosophila melanogaster* to humans (12, 16, 18, 27). This element functions as an inducible enhancer in mammalian cells (2) and consists of a symmetrical 14-base sequence containing 8 highly conserved bases (17). The heat shock element is found (26) centered at base −100 of the human heat shock protein 70 (hsp70) promoter discussed in this work, one of several hsp70 promoters in the human genome. This promoter is expressed in the absence of heat, and this basal expression is dependent on elements distinct from the heat shock element (27). When HeLa cells are incubated at 43°C, transcription from this promoter increases approximately 10-fold. Mutational analysis suggests that the heat shock element centered at base −100 is primarily responsible for this increase (27).

To detect a human factor that might be involved in heat shock induction, we prepared nuclear extracts from HeLa cells that were growing at 37°C and from cells that had been growing at 43°C for 1 h. We incubated increasing amounts of these extracts with a radiolabeled DNA fragment containing bases −148 to −74 of the human hsp70 promoter. Previous studies have shown that specific binding of a factor to a DNA fragment will retard the mobility of that fragment through a nondenaturing polyacrylamide gel (4, 6). When these reaction mixtures were subjected to electrophoresis through such a gel, several retained bands appeared in the lanes containing extracts from heat-shocked cells. The most prominent was characterized in detail (Fig. 1, band H). This band was present at much lower levels when extracts from normally growing cells were used. The amount of this band increased approximately linearly with increasing amounts of nuclear extract, indicating that the protocol provides a reliable estimate of the amount of the responsible factor in an extract. The level of this factor increased five- to sevenfold in extracts from heat-induced cells. This result compares favorably with the approximate 10-fold induction of this human hsp70 promoter upon heat shock (27).

To control for trivial differences between extracts in the ability to see sequence-dependent binding, we used a radiolabeled fragment containing bases −188 to −74 of the same human hsp70 promoter. This fragment contains the sequence CCAAT centered at base −149. Nuclear extracts contain a factor that binds specifically to this sequence (12a), and binding of this factor retards the mobility of DNA fragments through gels slightly less than binding of the heat-induced factor (R. E. Kingston, manuscript in preparation). The amount of CCAAT-binding factor was equivalent in heat-shocked and normal cells, in marked contrast to the results with the heat-induced factor (Fig. 1, band C).

We wished to determine what sequences were necessary for binding of the heat-induced factor. We attempted to inhibit binding by adding to the reaction unlabeled DNA fragments that either did or did not contain a heat shock element (Fig. 2). All fragments that contained this element successfully competed for binding, while fragments that contained other promoter elements did not (Fig. 2, band H). Notably, a fragment containing a *Drosophila melanogaster* heat shock element competed efficiently for binding. The bases of the heat shock elements represent the only homology between this promoter fragment and the human hsp70 promoter fragment, suggesting that this sequence is responsible for the observed interaction.

To characterize the binding interaction more precisely, we performed methylation interference studies (22). A methylated fragment containing bases −148 to −74 was incubated with nuclear extract prepared from heat-shocked cells. Bound and free fragments were separated by nondenaturing polyacrylamide gel electrophoresis. Analysis of the G residues that were N-7 methylated in each fraction indicated that methylation of three G residues on the plus strand and two G residues on the minus strand inhibited binding of the heat-induced factor (Fig. 3). These bases are all included within the 14-base heat shock element. While the eight highly conserved nucleotides within this element are symmetrical, binding was not completely symmetrical. Methylation of a G at base −94 inhibited binding, while methylation of the symmetrical G opposite base −107 did not inhibit binding.

* Corresponding author.

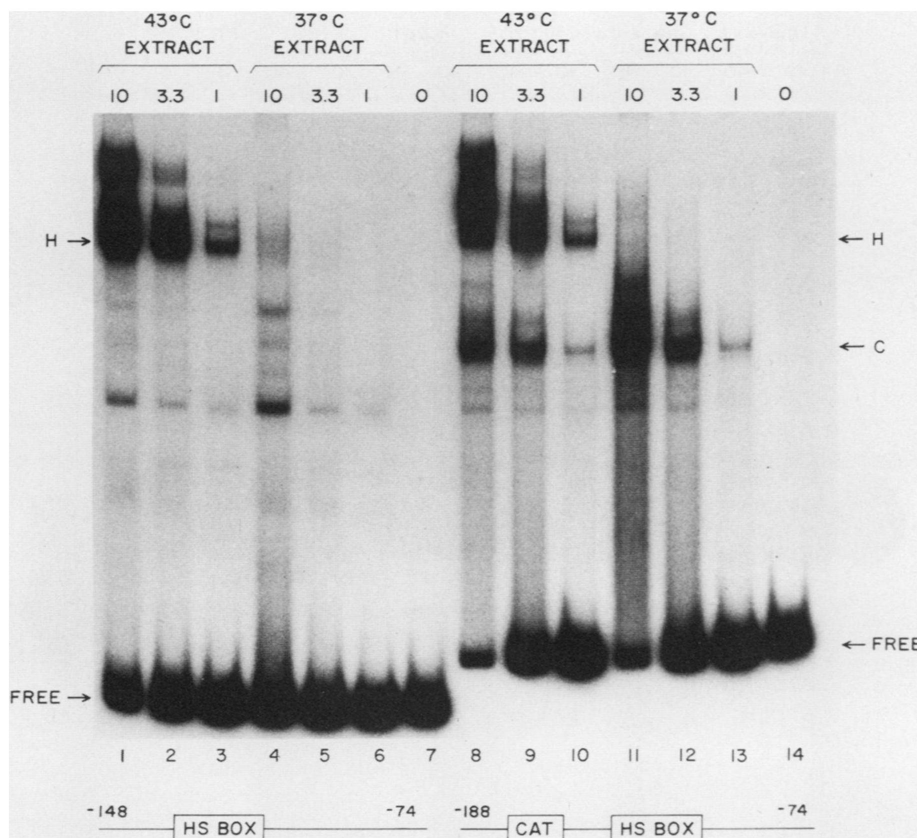

FIG. 1. Heat-shocked extracts contain increased amounts of a factor binding in the region of the heat shock element. Nuclear extracts were prepared from 1 liter of HeLa cells by the method of Dignam et al. (3) immediately after 1 h of incubation at 43°C (43°C extract, lanes 1 to 3 and 8 to 10) or from cells growing normally at 37°C (37°C extract, lanes 4 to 6 and 11 to 13). The indicated amounts of extract (lanes 1, 4, 8, and 11 10 μg of protein; lanes 2, 5, 9, and 12 3.3 μg of protein; lanes 3, 6, 10, and 13 1 μg of protein; and lanes 7 and 14, 0 μg of protein) were incubated with an end-labeled DNA fragment for 30 min at 30°C and subjected to electrophoresis on a 4% nondenaturing polyacrylamide gel. The labeled fragment contained bases −148 to −74 (lanes 1 to 7) or bases −188 to −74 (lanes 8 to 14) of the human hsp70 promoter. Binding reaction mixtures (20 μl) contained 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9), 12% (vol/vol) glycerol, 60 mM KCl, 0.12 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.3 mM dithiothreitol, 2 mM $MgCl_2$, 100 μg of poly(dI-dC) · poly(dI-dC) per ml, approximately 0.2 ng of the indicated labeled DNA, and the indicated amount of extract protein. H, Heat-induced factor; C, CAAT-binding factor; FREE, unbound DNA; HS BOX, location of the heat shock element; CAT, location of the sequence CCAATC.

The binding interaction was characterized further by DNase I footprinting (5). Binding reaction mixtures were briefly treated with DNase I immediately before separation on a preparative nondenaturing polyacrylamide gel. Comparisons of the bound and free fractions indicated that approximately 25 nucleotides were partially protected from DNase (Fig. 3). This protected region was centered slightly to one side of the symmetrical eight-base heat shock element. The DNase protection pattern was offset in the same direction relative to the heat shock element as the methylation interference pattern.

How rapidly does heat induce the ability of this factor to bind DNA? Nuclear extracts were prepared from HeLa cells that had been incubated at 43°C for various amounts of time, and equal amounts of extracted protein were analyzed by using the gel retention assay described above (Fig. 4). The level of the heat-induced factor was near maximal at 20 min and did not change significantly through 2 h of heat shock induction (Table 1). In contrast, the level of the CCAAT-binding factor decreased slightly over this time.

The rapid induction of the ability of this factor to bind argues against de novo synthesis of the factor and in favor of modification of a pre-existing factor. To test directly whether protein synthesis is necessary for activation of the factor, we incubated HeLa cells in 25 μg of cycloheximide per ml for 45 min prior to heat shock. Although cycloheximide increased the amount of the heat-induced factor in normal cells, the level of this factor remained heat inducible, and the amount of heat-shocked cells was similar in the presence or absence of cycloheximide (Table 1). These data suggest a model in which heating the cells results in modification of the DNA-binding capability of a preexisting factor. Alternatively, the factor could be sequestered in an insoluble or nonnuclear form that is mobilized by heat shock.

We have detected a heat-inducible DNA-binding factor in human nuclear extracts that binds to the heat shock element of a human hsp70 promoter. Deletion of the sequence that binds the factor eliminates the heat inducibility of this promoter in transfected cells (27). These data suggest that this factor plays a direct role in stimulating transcription

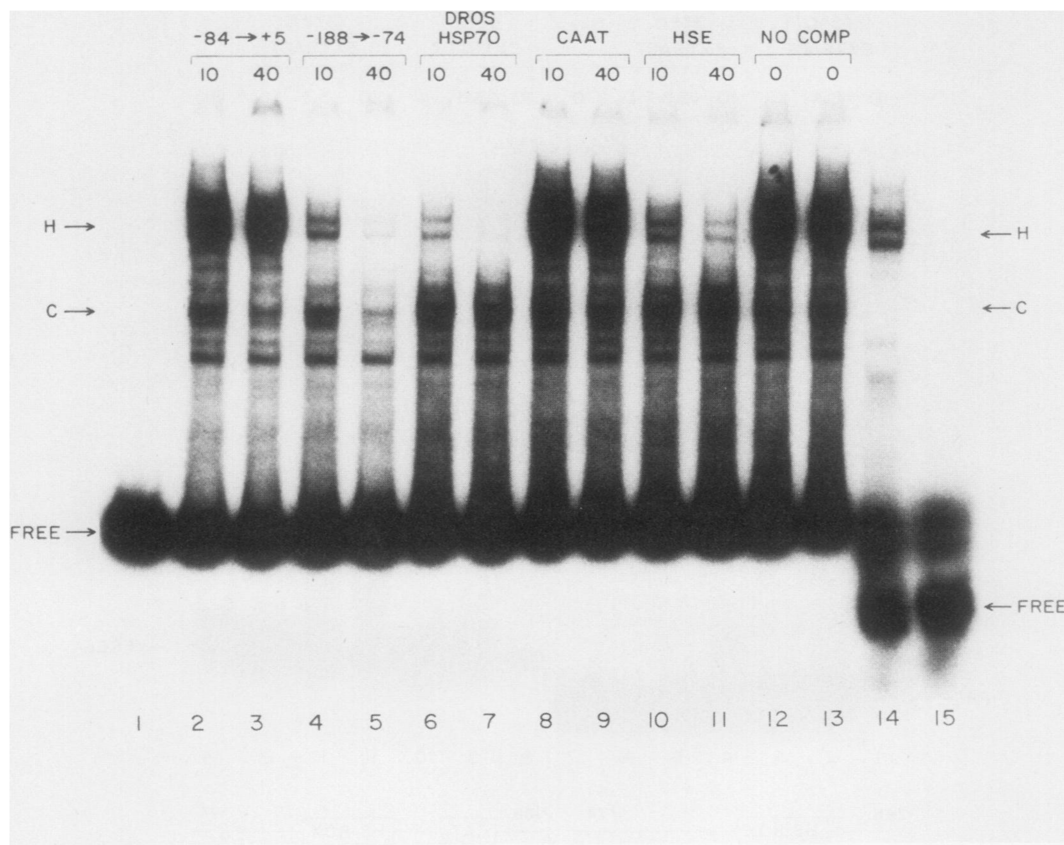

FIG. 2. Competition of the heat shock-specific band with fragments containing the heat shock element and nonspecific fragments. Nuclear extracts were prepared and incubations were done as described in the legend to Fig. 1 (lanes 2 to 13, 3 μg of protein; lanes 1 and 15, 0 μg of protein; and lane 14, 6 μg of protein). Extracts were incubated with approximately 0.4 ng of an end-labeled DNA fragment containing bases −188 to −74 of the human hsp70 promoter (lanes 1 to 13) or approximately 0.8 ng of a 40-base-pair synthetic oligonucleotide containing the heat shock element (lanes 14 and 15). Incubation mixtures also contained nonradioactive competitor DNA (in a 10- or 40-fold molar excess as indicated): lanes 1 and 12 to 15, no competitor; lanes 2 and 3, bases −85 to +5 of the hsp70 gene (no heat shock element); lanes 4 and 5, bases −188 to −74 of the hsp 70 gene (heat shock element at base −100); lanes 6 and 7, a 310-base-pair fragment of a *D. melanogaster* heat shock gene containing three heat shock elements (*Xho*I [base −200] to *Eco*RI [base +110], of plasmid pSP6-HS-9 [30]), derived from cloned fragment 232 described by Holmgren et al. (10); lanes 8 and 9, a 39-base-pair double-stranded synthetic oligonucleotide containing the CAAT sequence (top strand, 5′-CA CCG TCG ATT TCC CTT CTG AGC CAA TCA CCG AGC TCG A); and lanes 10 and 11, a 36-base-pair double-stranded synthetic oligonucleotide containing the heat shock element (bases −115 to −80 of the human hsp70 promoter). DNA concentrations were estimated by agarose gel electrophoresis.

from human heat shock promoters. Purification of the factor will be necessary to test this hypothesis.

A factor with similar characteristics has been detected in *D. melanogaster* (15, 28, 29). It is not clear at this time to what extent the drosophila and human factors are similar. Extensive alkylation interference studies characterizing the binding of the drosophila heat shock transcription factor to its binding site show a marked similarity to the studies presented here on the human factor (21). When one binding site is used, both factors show somewhat asymmetrical recognition of the palindromic heat shock element. The drosophila factor binds to two adjacent sites in the drosophila hsp70 promoter region, the more distal of which has a significantly lower binding affinity (23). In addition to the good heat shock recognition consensus sequence centered at base −100 of the human hsp70 promoter, there are two sequences with limited homology (5 of 8 bases match the 8-base consensus sequence) centered at bases −86 and −110. When nuclear extracts from heat-shocked cells were used in the retention assay, we saw three bands representing binding to this region (Fig. 1). The major band represented

TABLE 1. Activity of the human heat-induced factor in nuclear extracts of heat-shocked and non-heat-shocked HeLa cells[a]

Time (min) at 43°C	Amt of heat-induced factor in extracts in expt:		
	1	2	
		Cycloheximide absent	Cycloheximide present
0	1.0	1.0	3.7
20	5.4	ND	ND
60	6.3	7.4	13.3
120	7.1	ND	ND

[a] Nuclear extracts were prepared from 1 liter of HeLa cells immediately after incubation at 43°C for the indicated times. Activity was assayed as described in the legend to Fig. 1, and the values reported reflect the slope of the linear plot through a three-point concentration curve. Points on the curve were determined by cutting the band labeled H in Fig. 1 out of a dried 4% nondenaturing polyacrylamide gel and counting in a scintillation counter. The amount of heat-induced factor in the non-heat-shocked extract was arbitrarily assigned a value of 1.0. Cycloheximide, when present, was added to 25 μg/ml 45 min prior to the temperature shift. Cycloheximide inhibited 97% of protein synthesis in the experiment presented as measured by the incorporation of [^{35}S]methionine into an acid-insoluble form. ND, Not done.

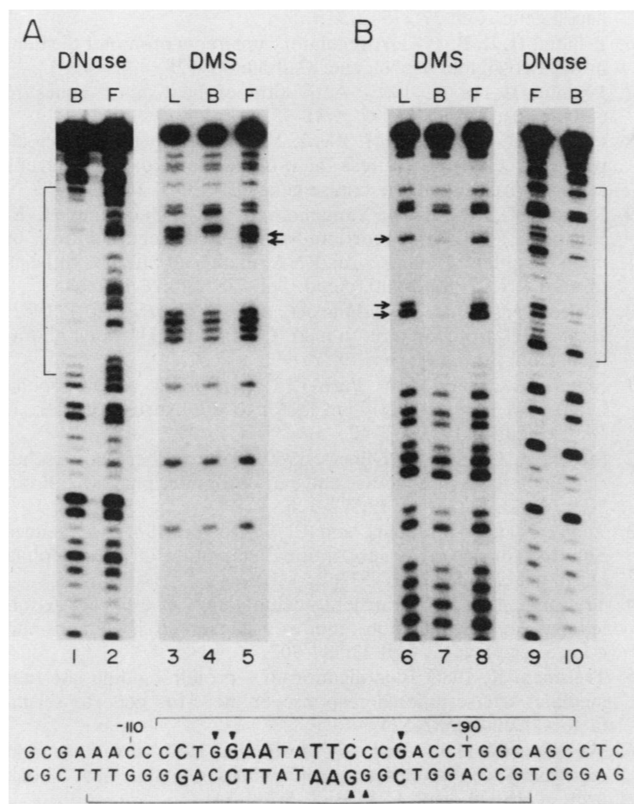

FIG. 3. Characterization of a human factor binding to the heat shock element. Methylation interference (DMS, lanes 3 to 8) and DNase protection (DNase, lanes 1, 2, 9, and 10) analyses of binding to the heat shock element were performed with label on either the bottom (A) or top (B) strand. Methylation-specific cleavage of DNA is represented as follows: lanes 3 and 6, DNA not incubated with extract (G ladder); lanes 4 and 7, DNA retained on a nondenaturing gel; lanes 5 and 8, free DNA from the retention gel. DNase protection is represented as follows: lanes 1 and 10, bound DNA after DNase treatment; lanes 2 and 9, free DNA after DNase treatment. DNase samples in each panel were run on the same gel as the methylation samples, so the ladder reaction (lanes 3 and 6) provides size markers for the DNase protection. The sequence of the human hsp70 promoter used is shown at the bottom of the figure. Large letters indicate the heat shock consensus sequence (C--GAA--TTC--G) (17). Arrows indicate G residues underrepresented in bound DNA in the methylation interference studies; brackets summarize DNase protection boundaries. In panel A, binding reactions (100 μl) were done as described in the legend to Fig. 1 but the mixtures contained 3 ng of a fragment containing bases −148 to −74 and 16 μg of extract. In panel B, reaction mixtures contained 8 ng of a fragment containing bases −148 to −74 and 16 μg of extract. Bound and free fragments were eluted and analyzed as described by Gilman et al. (7).

binding to the sequence centered at base −100 (Fig. 3). We have been unable to determine whether the other bands represent a complex that binds to the adjacent sequences.

Mammalian cells respond to numerous environmental signals by rapidly increasing the expression of a set of specific genes. Of fundamental importance is the mechanism by which the stimulatory signal affects gene expression. A recently characterized example that is similar in some aspects to heat shock induction is the stimulation of c-*fos* transcription observed immediately after the exposure of cells to certain growth factors (9, 11, 13). The sequence

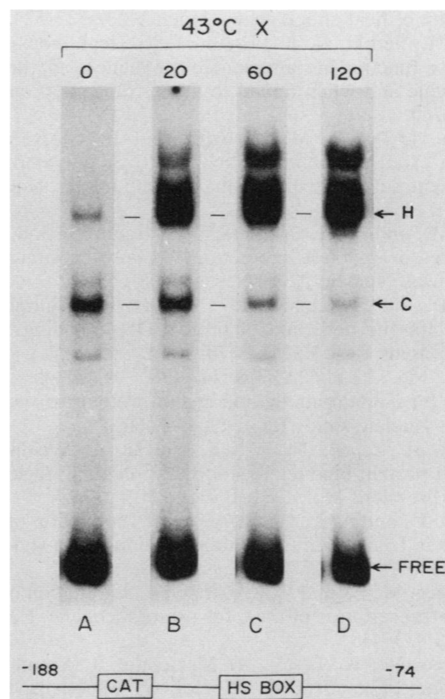

FIG. 4. Time course of induction of the heat-induced human factor. HeLa cells (1 liter) were incubated for the indicated times at 43°C, and nuclear extracts were immediately prepared. Extract protein (6 μg) was analyzed by the gel retention assay described in the legend to Fig. 1. An end-labeled fragment spanning bases −188 to −74 was used in the experiment. Abbreviations as for Fig. 1.

responsible for this stimulation has been mapped, and a factor that binds to it has been detected (7, 24, 25). In this instance, the binding activity of the protein does not appear to be increased after stimulation by growth factors, suggesting that regulation involves other components.

It has been proposed that the induction of the heat shock response involves the activation of a preexisting factor via a mechanism involving denatured proteins (1, 8, 14). The data reported here lend experimental support to this model and suggest that the activated human factor binds directly to the heat shock element. In contrast to the stimulation of c-*fos* expression, the ability of the factor to bind to the heat shock element in a sequence-specific manner is altered by exposure to heat. The activation of a preexisting receptor protein is responsible for the induction of the mouse mammary tumor virus promoter by glucocorticoids, which also occurs with rapid kinetics (19, 31). These observations imply that the rapid induction of mammalian gene expression can occur by multiple mechanisms. The data presented here suggest that human nuclear extracts may be used to develop in vitro assays for the activation of this mammalian factor, thus allowing dissection of the mechanism of heat shock induction in human cells.

We are grateful to M. Gilman and L. Chodosh for technical suggestions, R. Hyde for preparing the manuscript, L. Kizilay and J. Smith for preparing the synthetic oligonucleotides, and D. Moore and M. Gilman for critical comments on the manuscript.

This work was supported by a grant from Hoechst AG.

LITERATURE CITED

1. **Ananthan, J., A. L. Goldberg, and R. Voellmy.** 1986. Abnormal proteins serve as eukaryotic stress signals and trigger the

activation of heat shock genes. Science **232**:522–524.
2. **Bienz, M., and H. R. B. Pelham.** 1986. Heat shock regulatory elements function as an inducible enhancer in the Xenopus hsp70 gene and when linked to a heterologous promoter. Cell **45**:753–760.
3. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
4. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6506–6525.
5. **Galas, D., and A. Schmitz.** 1978. DNAse footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res. **5**:3157–3170.
6. **Garner, M. M., and A. Revzin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions. Nucleic Acids Res. **9**:3047–3060.
7. **Gilman, M. Z., R. N. Wilson, and R. A. Weinberg.** 1986. Multiple protein-binding sites in the 5'-flanking region regulate c-*fos* expression. Mol. Cell. Biol. **6**:4305–4316.
8. **Goff, S. A., and A. L. Goldberg.** 1985. Production of abnormal proteins in E. coli stimulates transcription of lon and other heat shock genes. Cell **41**:587–595.
9. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) **311**:433–438.
10. **Holmgren, R., K. Livak, R. Morimoto, R. Freund, and M. Meselson.** 1979. Studies of cloned sequences from four Drosophila heat shock loci. Cell **18**:1359–1370.
11. **Kruijer, W., J. A. Cooper, T. Hunter, and I. M. Verma.** 1984. PDGF induces rapid but transient expression of the c-fos gene. Nature (London) **312**:711–716.
12. **Mirault, M.-E., R. Southgate, and E. Delwart.** 1982. Regulation of heat shock genes: a DNA sequence upstream of Drosophila hsp70 genes is essential for their induction in monkey cells. EMBO J. **1**:1279–1285.
12a. **Morgan, W. D., G. T. Williams, R. I. Morimoto, J. Greene, R. E. Kingston, and R. Tjian.** 1987. Two transcriptional activators, CCAAT-box-binding transcription factor and heat shock transcription factor, interact with a human hsp70 gene promoter. Mol. Cell. Biol. **7**:1129–1138.
13. **Muller, R., R. Bravo, J. Burckhardt, and T. Curran.** 1984. Induction of the c-fos gene and protein by growth factors precedes activation of c-myc. Nature (London) **312**:716–720.
14. **Munro, S., and H. Pelham.** 1985. What turns on heat shock genes? Nature (London) **317**:477–478.
15. **Parker, C. S., and J. Topol.** 1984. A Drosophila RNA polymerase II transcription factor binds to the regulatory site of an hsp70 gene. Cell **37**:273–283.
16. **Pelham, H. R. B.** 1982. A regulatory upstream promoter element in the Drosophila hsp70 gene. Cell **30**:517–528.
17. **Pelham, H. R. B.** 1985. Activation of heat-shock genes in eukaryotes. Trends Genet. **1**:31–35.
18. **Pelham, H. R. B., and M. Bienz.** 1982. A synthetic heat-shock promoter element confers heat-inducibility on the herpes simplex virus thymidine kinase gene. EMBO J. **1**:1473–1477.
19. **Ringold, G. M., K. R. Yamamoto, J. M. Bishop, and H. E. Varmus.** 1977. Glucocorticoid-stimulated accumulation of mouse mammary tumor virus RNA: increased rate of synthesis of viral RNA. Proc. Natl. Acad. Sci. USA **74**:2879–2883.
20. **Schlesinger, M. J., M. Ashburner, and A. Tissieres (ed.).** 1982. Heat shock from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Shuey, D. J., and C. S. Parker.** 1986. Binding of Drosophila heat-shock gene transcription factor to the hsp70 promoter. J. Biol. Chem. **261**:7934–7940.
22. **Siebenlist, U., and W. Gilbert.** 1980. Contacts between Escherichia coli RNA polymerase and early promoter phage T7. Proc. Natl. Acad. Sci. USA **77**:122–126.
23. **Topol, J., D. M. Ruden, and C. S. Parker.** 1985. Sequences required for *in vitro* transcriptional activation of a Drosophila hsp70 gene. Cell **42**:527–537.
24. **Treisman, R.** 1985. Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. Cell **42**:889–902.
25. **Treisman, R.** 1986. Identification of a protein-binding site that mediates transcriptional response of the c-fos gene to serum factors. Cell **46**:567–574.
26. **Wu, B., C. Hunt, and R. Morimoto.** 1985. Structure and expression of the human gene encoding major heat shock protein HSP70. Mol. Cell. Biol. **5**:330–341.
27. **Wu, B. J., R. E. Kingston, and R. I. Morimoto.** 1986. Human HSP70 promoter contains at least two distinct regulatory domains. Proc. Natl. Acad. Sci. USA **83**:629–633.
28. **Wu, C.** 1984. Two protein-binding sites in chromatin implicated in the activation of heat-shock genes. Nature (London) **309**:229–234.
29. **Wu, C.** 1985. An exonuclease protection assay reveals heat-shock element and TATA box DNA-binding proteins in crude nuclear extracts. Nature (London) **317**:84–87.
30. **Wurm, F. M., K. A. Gwinn, and R. E. Kingston.** 1986. Inducible overproduction of the mouse c-myc protein in mammalian cells. Proc. Natl. Acad. Sci. USA **83**:5415–5418.
31. **Yamamoto, K. R.** 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19**:209–252.